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Mangiferin protects against 1-methyl-4-phenylpyridinium toxicity mediated by oxidative stress in N2A cells

Laïla Amazzal, Agnès Lapôtre, Frédéric Quignon, Denyse Bagrel *

Laboratoire d'Ingénierie Moléculaire et Biochimie Pharmacologique, FR CNRS 2843, UFR SciFA, Université Paul Verlaine-Metz, Rue du Général Delestraint, 57070 Metz, France

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This work is dedicated to Dr Alain Minn who devoted part of his life to the study of drug metabolizing enzymes in the brain.

Abstract

1-Methyl-4-phenyl-pyridine ion (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a Parkinsonian syndrome in humans and animals, a neurotoxic effect postulated to derive from oxidative stress. We report here the first investigation of MPP^+-induced oxidative stress in the murine neuroblastoma cell line N2A. Significant cell death was observed following exposure to 0.25 mM MPP^+. Markers of oxidative stress included decreased intracellular levels of GSH after 48 h of exposure (85% depletion) as well as an increase in GSSG. Expression of both superoxide dismutase 1 (*sod1*) and catalase (*cat*) mRNA was increased, as well the activity of catalase. These cellular effects were, at least partially, reversed by treatment with the natural polyphenol mangiferin. Administration of mangiferin protected N2A cells against MPP^+-induced cytotoxicity, restored the GSH content (to 60% of control levels), and down-regulated both *sod1* and *cat* mRNA expression. Together, these results suggest that the protective effect of mangiferin in N2A cells is mediated by the quenching of reactive oxygen intermediates. Therefore, mangiferin could be a useful compound in therapies for degenerative diseases, including Parkinson's disease, in which oxidative stress plays a crucial role.

Keywords: Oxidative stress; Glutathione; Mangiferin; N2A; MPP^+

In Parkinson's disease (PD) the nigral level of iron is increased and may contribute to the hyperproduction of reactive oxygen species (ROS), leading to oxidation and nitration of proteins, lipids and DNA observed in post-mortem brains [9]. However, the primary causes of cell death remain controversial, and insights into the pathogenesis of PD obtained from experimental models of the disease continue to be valuable [7]. For example, the synthetic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes a Parkinsonian syndrome in humans and animals. Its active metabolite, 1-methyl-4-phenyl pyridinium (MPP^+), induces the formation of ROS, such as superoxide or hydroxyl radicals [4], suggesting that oxidative stress underlies the neurotoxicity of MPTP. MPTP-based animal models are therefore useful for identifying compounds which mitigate the effects of ROS, and thus have promise as neuroprotective agents for the treatment of PD.

The potential utility of a number of antioxidants (e.g. vitamins C and E, polyphenols, melatonin . . .) has been proposed on the basis of epidemiological studies. Here, we aimed to evaluate the protective capacity of mangiferin (1,3,6,7-tetrahydroxy-2-beta-D-(glucopyranosyl)-xanthen-9-one) against MPP^+ toxicity. Mangiferin is the primary polyphenol component of Vimang. Its antioxidant properties have been extensively evaluated in various cells, including neurons [23], and likely underlie its diverse pharmacological activities [13]. As mangiferin is able to traverse the blood–brain barrier [16], it has real potential to ameliorate the oxidative stress observed in neurodegenerative disorders. Despite these promising features, to our knowledge, the potential capacity of mangiferin to counteract the oxidative stress mediated by PD-inducing toxins has never been tested to date. Moreover, as increased iron levels are observed in *nigra* of MPTP treated animals, iron is probably involved in MPP^+ toxicity. Thus, the interest in testing mangiferin is two-fold: first, it acts as a radical scavenger and second, it inhibits ROS production through the Fenton and Haber-Weiss reactions. We have therefore investigated the ability of mangiferin

* Corresponding author. Tel.: +33 3 87378404; fax: +33 3 87378423.
E-mail address: bagrel@univ-metz.fr (D. Bagrel).

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to protect against the deleterious effects of MPP^+ in a cellular model.

To date, the rat pheochromocytoma PC12 cell line has often been selected as a model system for studying various aspects of MPTP-induced oxidative stress. We chose, however, to use the mouse neuroblastoma cell lines N2A, as N2A cells take up MPP^+ with high efficiency, and are more sensitive to the toxins than other cell lines [20]. This study reports the first assessment of MPTP/MPP^+ -induced oxidative stress in this cell line. In order to judge the oxidative stress induced by MPP^+ in the presence and absence of antioxidants, we focused our analysis on glutathione homeostasis. Glutathione is synthesized by the consecutive action of gamma-glutamyl-cysteinyl-ligase (GCL) and glutathione synthetase. In the majority of mammalian cells, reduced glutathione (GSH) acts as a major cellular antioxidant. Indeed it serves as an electron donor in reactions catalyzed by glutathione peroxidase (GPX). GPX reduces hydroperoxides into the corresponding alcohols, yielding glutathione disulfide (GSSG), which is in turn a substrate for glutathione reductase (GSSR). This NADPH-dependent reaction regenerates GSH, the only active form of the tripeptide. Inside cells, GSH can also be consumed by direct non-enzymatic reactions involving radicals, and by conjugation with electrophilic compounds, a reaction catalyzed by glutathione transferases (GSTs) [10]. Importantly, it has been shown that dopaminergic cells are particularly vulnerable to oxidative stress and that reduced levels of GSH in the *substantia nigra pars compacta* contributes to the pathogenesis of PD [7,19].

Unless specifically indicated, chemicals were purchased from Sigma, France, and cell culture medium and additives from Eurobio, France. N2A cells (a generous gift from "INSERM U706", France) were grown in low glucose (1 g/L) DMEM medium, supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin at 37 °C in 5% CO_2. To carry out cellular toxicity, glutathione, enzymatic activity and gene expression determinations, cells were exposed to MPP^+ 24 h after seeding. Neuroprotective effects were evaluated by adding the antioxidant (100 μM mangiferin, 100 μM trolox or 1 μM resveratrol) to the culture medium 6 h after seeding, and for 18 h prior to exposure to 0.25 mM MPP^+. MPP^+ treatment was continued for an additional 24 or 48 h, in the presence of the antioxidant.

Cell viability was assessed by trypan blue exclusion test (adherent and suspended cells were counted) and MTT assay [18]. Determination of the end points and the median effective concentration (EC_{50}) were performed using generalized linear models, as described in [17]. After 12, 24, 36, or 48 h of treatment, cells were harvested and the intracellular content of both reduced (GSH) and oxidized (GSSG) forms of glutathione was determined [21]. GSSR, GST, and catalase (CAT) activities were respectively determined by the methods of Carlberg and Mannervick [6], Habig et al. [8], and Beers and Sizer [2]. Proteins were quantified using the Lowry et al. [15] method. ROS were detected as described in [21], and the antioxidant potential of the tested compounds was assessed using the *in vitro* FRAP (ferric reducing ability of plasma) method [3].

mRNA levels were estimated semi-quantitatively using a reverse transcription polymerase chain reaction (RT-PCR) assay [14]. The specific forward/reverse primers used were: *gssr*: 5′-tcagttggcatgtcatcaag-3′/5′-tgcagttggaactgatgag; *gpx1*: 5′-atgtgtgctgctcggctctc-3′/5′-tgctgggacagcagggtttc-3′; *sod1*: 5′-atggcgatgaaagcggtgtg-3′/5′-gcgcaatcccaatcactcca-3′; *cat*: 5′-aggtttggcctcacaaggac-3′/5′-gcggtagggacagttcacag-3′; *gcl:* 5′-gagctgggaagagacccagc-3′/5′-cgggggtgcttgtttatggc-3′; *gsta3*: 5′-atggcggggaagccagtcct-3′/5′-agttgtccacaacgcccggg-3′; and *actb* (β-actin reference): 5′-atggatgacgatatcg-ctgc-3′/5′-ttctccatgtcgtcccagtt-3′. All experiments were performed in triplicate at least, and data are expressed as mean ± S.D. Comparisons of results were performed using the Student's *t*-test.

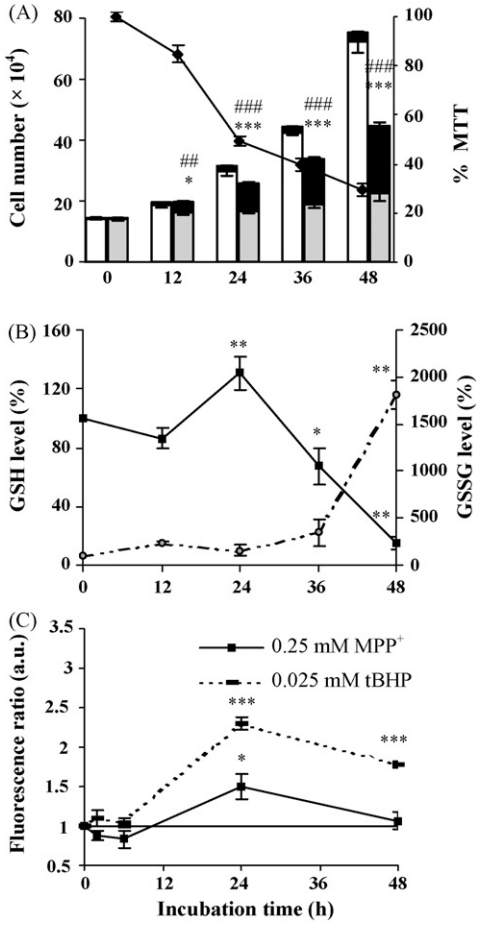

Fig. 1. Toxicity and effects of MPP^+ on the redox status of N2A cells. (A) Kinetics of toxicity of MPP^+ assessed by trypan blue and MTT assays. White and grey bars: viable cells of respectively control and MPP^+-exposed cells; black bars: dead cells. $^{*}p<0.05$ and $^{***}p<0.001$, viable MPP^+-treated compared to control cells; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ dead MPP^+-treated compared to control cells. (B) Intracellular level of GSH (continuous line) and GSSG (dotted line) in N2A cells exposed to 0.25 mM MPP^+. Data are expressed as percentage of glutathione level (exposed cells vs. control cells). The control values for GSH (100%) were 96 ± 6, 50 ± 3, 59 ± 2, and 34 ± 4 nmol/mg proteins for 12, 24, 36, and 48 h exposure, respectively. The control values for GSSG (100%) were 0.1 and 0.3 nmol/mg proteins. (C) ROS production. tBHP was used as a positive control. For each time point, the fluorescence ratio of the exposed cells/time-matched control is given. For (B) and (C), $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$, compared to control. (A), (B) and (C): mean ± S.D.; $n=3$.